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Original Article

Ectopic expression of telomerase enhances osteopontin and osteocalcin expression during osteogenic differentiation of human mesenchymal stem cells from elder donors

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Abstract

Age related bone loss is one of the most prevalent diseases in the elder population. The osteoblasts are the effectors cells of bone formation and regeneration. With the aging the osteoblasts become senescent reducing their ability to produce bone. Cellular replicative senescence is triggered by telomers shortening. Telomerase elongate the telomers length and maintain the cell proliferative capacity. Here, we demonstrated that the expression of human telomerase reverse transcriptase mediated by an adenovirus vector increases the levels of osteopontin and osteocalcin mRNA during the *in vitro* osteogenic differentiation of elderly human mesenchymal stem cells. Bone marrow human mesenchymal stem cells were obtained from old donors (>65 years) and induced to differentiate into osteoblasts for 14 days. The levels of mRNA of human telomerase reverse transcriptase, osteopontin and osteocalcin during the differentiation were assessed by semi-quantitative PCR before and during the differentiation on days 7 and 14. Infected cells showed 1.5 fold increase in telomerase expression. Also *telomerized* cells exhibit 1.5 fold increase in osteopontin and 0.5 fold increase in osteocalcin expression compared to primary osteoblasts isolated from the same donors. The transformed cells were not able to form tumours in NUDE mice.

Key words: telomerase, human mesenchymal stem cells, osteogenesis

Introduction

Osteoporosis is one of the most prevalent diseases in the elder population. The age related bone loss is multifactorial phenomena whose mechanisms are still not fully elucidated. The bone tissue homeostasis is orchestrated by osteoblasts and osteoclasts whose function is to maintain the balance between deposition and re-absorption of bone matrix. In age-related bone loss the balance is shifted due to a deficit in osteoblast function accompanied by an increase in osteoclast activity[1]. The decrease in bone forming capacity in senile osteoporosis is

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mediated by defects in the number and function of osteoblasts; a possible causative mechanism of this is the senescence of osteoblasts and their precursors[2]. Studies on mice have shown that age-related osteoporosis is the result of impaired osteoblast differentiation associated with cell senescence of mesenchymal stem cells[3]. It is a possibility that human mesenchymal stem cells undergo a process of aging in vivo, leading to diminished ability to form and maintain bone homeostasis with age[4].

The osteogenic potential of bone marrow mesenchymal stem cells makes them ideal candidates for use in bone tissue repair[5,6,7]. Unlike most other human adult stem cells, mesenchymal stem cells can be obtained in enough quantities for therapeutic use. However in old donors where the aging process results in an intrinsic increase in bone marrow fat followed by a reduction in the number of marrow cells, the number of mesenchymal stem cells is compromised. This problem can be overcome by the ex vivo expansion of donor's cells until they reach an amount necessary for clinical applications. Although, it was reported that human mesenchymal stem cells growing in vitro cease dividing at around 40-50 population doublings[8].

In somatic cells, the number of divisions is associated with a shortening of telomere size leading to a decrease in the proliferative state. The telomerase activity can prevent the loss of the telomers and subsequent reduction of proliferation in vitro[9,10]. In an attempt to rescue the cells from replicative senescence the telomerase subcatalytic unit (hTERT) was introduced in mesenchymal stem cells. After the introduction of the hTERT, the number of passages increased to 260 and the cells still exhibited undifferentiated phenotype and preserved multilineage potential[11]. Besides, ectopic expression of telomerase enhances osteoblast differentiation and increases the capacity of bone formation[12]. Altogether, these studies provide strong evidence that telomerase or telomere status is a critical component of bone formation. Here we show that the ectopic expression of hTERT promotes increase of osteopontin and osteocalcin levels during the osteogenic differentiation of human mesenchymal stem cells from old donors.

Methodology

hMSC and primary osteoblasts isolation and culture

Human mesenchymal stem cells and fragments of bone tissue were obtained from three donors (>65 years) undergoing orthopaedic surgery. The donors didn't have records or apparent involvement with bone metabolism diseases. The protocols and the consent forms were approved by the local Ethical Committee.

Mesenchymal stem cells from iliac crest bone marrow aspirates were collected in sterile heparin tubes and separated by Ficoll-Hypaque gradient to yield mononuclear cells. Cells were plated and expanded in non-inductive medium, Dulbecco's modified Eagle's medium high glucose (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin and streptomycin (Gibco) in a humidified 95% air 5% CO_2 incubator at 37°C. Non-adherent cells were removed after 3 days. After 7 days the cells gave rise to a monolayer culture. The medium was changed every 2 days and when the cells reached approximately 90% confluence, they were split in ratio of 1:5 by enzymatic dissociation using 0.25% trypsin-ETDA (Gibco). The cells were expanded for five passage doublings and then induced to differentiate.

Primary osteoblasts were isolated from iliac crest fragments. Briefly, the fragments were subjected to three sequential incubations of 15 minutes in trypsin-EDTA, followed by three sequential incubations of 10 minutes in collagenase (Gibco). The cells were collected by centrifugation (1400 rpm for 10 minutes) from the second and third collagenase bath

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and maintained in non-inductive medium in a humidified 95% air 5% CO_2 incubator at 37◦C.

Osteogenic differentiation of human mesenchymal stem cells

Human mesenchymal stem cells were dissociated by trypsin and seeded at the concentration of $2x10^5$ cells/ well in 6 well plates. The differentiation was induced by adding to the non-inductive medium 0.01M of dexamethasone (Sigma), 50 M of ascorbic acid (Sigma) and 10mM of glycerophosphate (Sigma). The medium was changed twice a week. The cells were differentiated for two weeks.

Flow cytometry analysis of hMSC

Cells at passage doubling number five were harvested with trypsin-EDTA, centrifuged at 1500 g for 5 min, and re-suspended in 0.1% BSA (Sigma) in phosphate-buffered saline. At least 500,000 cells (in 100 µl PBS/ 0.5% BSA) were incubated with primary monoclonal antibodies or respective isotype controls for 30 minutes at 4°C. After washing steps, the cells were incubated with fluorescent conjugated antibodies for 30 minutes at 4°C, washed again and then fixed in 2% paraformaldehyde in PBS. The cells were analysed by flow cytometry using a FACScan (Becton Dickinson). All data were interpreted using CellQuest software. The antibodies used were mouse IgG1 anti- human CD29 (Santa Cruz Biotechnology), mouse IgG1 anti-human CD44 (Santa Cruz Biotechnology), mouse IgG3 anti-human CD34 PE conjugated (Santa Cruz Biotechnology), rabbit anti-mouse IgG1 FITC conjugated (Calbiochem).

Semi-quantitative

RT-PCR Mesenchymal stem cells and osteoblasts were obtained from three donors as described before. The expression levels of hTERT, osteopontin and osteocalcin were compared between infected and non-infected hMSC subjected to osteogenic differentiation for 14 days. Undifferentiated hMSC were used as relative comparison for the expression of hTERT level and primary human osteoblasts were used as relative comparison for the osteopontin and osteocalcin levels. Before start the differentiation process and at days 7 and 14, the total RNA was extracted from the samples described above with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To exclude genomic DNA contamination, the RNA was treated with RNase free DNase I (Invitrogen) prior the reverse transcriptase amplification. After the DNase digestion, 0.5 g of total RNA was reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen) and oligo-dT primer according to the manufacturer's instructions. For semi-quantitative PCR the number of cycles was previously optimized (data not shown). The conditions were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification protocol. One Micro L of cDNA product was amplified in 50 Micro L of reaction by 1 unit of Taq DNA polymerase (Promega) with 10 pmol of each primer (hTERT, HPRT, osteopontin or osteocalcin) and 0.2 mM dNTPs in the buffer with MgCl2 provided by the manufacturer. After 3 minutes at 95°C, the amplification was carried out for 20 cycles, each of 30 seconds at 92◦C, 30 seconds at 58°C and 45 seconds at 72°C. Each sample was processed in triplicate. Non-reverse transcribed RNA was also subjected to PCR to confirm the absence of genomic DNA. The expected sizes for osteopontin, osteocalcin, hTERT and HPRT PCR products were 283, 120, 152, 383 bp respectively. The PCR products were resolved by electrophoresis in 2% agarose gel; stained with ethidium bromide and visualized under ultraviolet light system. The densitometric analysis of the gel bands was performed using the ImageQuant TL software (GE Healthcare). Housekeeping gene HPRT mRNA was used as control for variations in the input of RNA according with the following formula Rs= Ds X Dc, where Rs is value to gene mRNA analyzed (OP, OC and hTERT), Ds is the gene densitometric value and Dc is the HPRT densitometric value. Each change in gene expression is expressed as fold change and it

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is relative to undifferentiated hMSC or osteoblasts. A list of the genes analysed by semi-quantitative PCR and primer sequences can be found in Table 1.

Generation of recombinant Adenovirus-hTERT
The human reverse catalytic subunit cDNA was generously provided by Dr Robert Weinberg from Whitehead Institute for Biomedical Research, Massachusetts13. The hTERT from the pCI-neo plasmid was subcloned into the plasmid pShuttle (Clontech) between Nhe I (New England Biolabs) and Not I (New England Biolabs) sites (Figure 2). The fragment coding for hTERT was subsequently subcloned into the adenoviral vector pAdeno-X (Clontech) using I-Ceu I e PI-Sce I sites. To verify the absence of DNA rearrangement in the pAdeno-hTERT, the adenoviral vector DNA was digested with Xho I (New England Biolabs) generating a pattern that was crosschecked with the pattern generated in silico (Figure 2). HEK293A cell line (ATCC) was transfected by eletroporation with the linear viral DNA Ad-hTERT digested with Pac I (New England Biolabs) for the generation of viral particles. About 10 days after transfection, the cells were harvested and pelleted by low-centrifugation, and the viruses were liberated by three freeze/thaw cycles. The lysate containing the recombinant adenoviruses was then purified and amplified. To generate higher titre viral stocks, the HEK293A cells were infected by the cell lysate and the harvest process was repeated. The cell lysate was 0.22 m filtered and titulated by Spearman-Karber method.

Primary cell culture infection
The infection of the primary human osteoblasts and hMSC was carried out by incubating them with 0.22 µm filtered cell lysate, 5 pfu/ mL. The infecting medium was left in contact with the cells for 2 hours. The cells were then washed in PBS and fresh medium was added.

Analysis of hTERT expression by immunocytochemistry
Immunofluorescence was performed on primary osteoblasts 48 hours after the infection with Ad-hTERT. The osteoblasts were fixed with cold acetone for 5 min at -20°C, blocked for 2 hours at room temperature with phosphate-buffered saline containing 3% bovine serum albumin, 0.05% Tween and 0.2% TritonX-100, then labelled with primary antibody for 1h at room temperature. Osteoblasts were labelled with rabbit monoclonal antibody anti-Telomerase (Abcam) to confirm that infected osteoblasts expressed the intended protein. To facilitate the identification of the plasma membrane, the cells also were incubated with mouse monoclonal antibody anti- tubulin (Abcam). After primary antibody incubation the cells were rinsed with phosphate-buffered saline and incubated with goat anti-rabbit Alexa 488-conjugated secondary antibody (Molecular Probes) and goat anti-mouse Alexa 568-conjugated secondary antibody (Molecular Probes). Negative controls were labelled with secondary antibodies only. Specimens were examined using a BioRad MRC 1024 Confocal Microscope. To ensure specificity of staining, images were obtained using confocal machine settings at which no fluorescence was detectable in negative control samples. Specimens were serially excited at 488 nm and observed at 505~550 nm to detect Alexa 488, then excited at 568 nm and observed at 585 nm to detect Alexa 568.

In vivo tumorigenesis assay in NUDE mice
The mice were handled under a unidirectional laminar airflow hood. Non-infected hMSC and MDAMB-231 (breast cancer) cells were used as negative control and positive control, respectively, for tumour formation. The hMSC were grown in non-inductive medium, infected with Ad-hTERT and then subjected to osteogenic differentiation for 14 days. The cells were dissociated with trypsin, washed and resuspended in PBS, adjusted to a concentration of 1×10^7 cells/ 300uL/ animal and injected subcutaneously into the right flank of eight-week-old female athymic nude mice. Tumour development was followed in individual animals (three per group) during 60

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days. The animals had free access to food and water and were maintained in a room with a 12-hours light/ dark cycle under sterile conditions. The local Ethical Committee for Animal Care approved the animal experiment procedures.

Statistical analysis

The semi-quantitative PCR results are expressed as the mean + SD. The data were compared using analysis of variance (ANOVA) and Student's t-test and it is the average of three samples (3 donors) where each experiment was carried out three times and for each sample the PCR was performed in triplicate. It was considered statistically significant $p<0.05$.

Results

Flow cytometry

The hMSC grown as adherent monolayer cell culture for 21 days were analysed by FACS to confirm the presence of CD44 and CD29 positive cell population. To ensure the cells were not mixed with hematopoietic stem cells, the presence of the CD34+ cells was also evaluated. As shown on the figure 1, there's a complete absence of CD34+ cells assuring the sample was not mixed with hematopoietic stem cells. The same population were positive for CD29 (64%) and CD44 (94%), characteristic surface proteins expressed by human mesenchymal stem cells14.

Analysis of hTERT expression by immunocytochemistry

Immunofluorescence was carried out in primary human osteoblasts collected from elderly donors in order to detect if the cells infected by recombinant adenovirus-hTERT were expressing hTERT. Osteoblasts were chosen because primary cells usually don't express telomerase. The osteoblast culture was infected with 5 pfu/ mL of Adenovirus-hTERT. The infecting medium was left in contact with the cells for 2 hours. The cells were then washed in PBS and fresh medium was added. After 48 hours, immunofluorescence was performed to detect the expression of telomerase. As it can be observed in the figure 3, the osteoblasts infected by Ad-hTERT were expressing telomerase and, as expected, non-infected osteoblasts were not.

Semi-quantitative RT-PCR to osteopontin, osteocalcin and telomerase

To evaluate the effect of telomerase expression on the levels of osteopontin and osteocalcin, hMSC were infected 48 hours before the beginning of the differentiation. As osteopontin and osteocalcin are not present in hMSC, the levels of their expression were taken relative to osteoblasts collected from the same donors. And because osteoblasts do not express telomerase, the level of mRNA was compared to undifferentiated hMSC. Infected Ad-hTERT-hMSC and non-infected hMSC were induced to osteodifferentiation. The samples were amplified for consecutive cycles within the exponential phase of PCR reaction. The optimal numbers of PCR cycles determined were 20. The samples were compared with 20 cycles. As control, the extracted total RNA of each sample was subjected to PCR. No PCR products were detected indicating lack of genomic DNA in the samples (data not shown).

The osteogenic differentiation led to a reduction of telomerase mRNA (Figure 4B), while adenovirus-hTERT infected cells had 1.5 fold increase in telomerase expression (Figure 4A). The osteogenic process of human mesenchymal stem cells was influenced by expression of hTERT. Compared to osteoblasts, telomerized hMSC produces higher levels of osteopontin, 1.5 fold increase on 14th day of differentiation (figure 4C). There's no change in expression of osteopontin over 14 days in non-infected cells (Figure 4D). Although osteopontin is a normally expressed by osteoblasts, our results show that hMSC also express osteopontin and it may be the reason why the osteopontin expression was not significantly

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altered during differentiation of non-infected cells.

The osteocalcin expression was increased by 0.5 fold in telomerized cells (Figure 4E) and it was maintained until day 14. The level of osteocalcin in Ad-hTERT-hMSC was higher than in osteoblasts and it could be detected on 7th day of differentiation suggesting an enhancement of the osteogenic potential in those cells. In non-infected cells, the mRNA didn't reach the osteoblast's level (Figure 4F).

Tumorigenesis assay

The NUDE mice injected with MDAMB-231 (Figure 5A) developed tumours within 7 days and had to be sacrificed after 28 days due the tumour size. The mice injected with hMSC (Figure 5B) and differentiated Ad-hTERT-hMSC (Figure 5C) didn't develop any tumours. The mice were followed for 60 days and then sacrificed and dissected. No evident signs of tumour were found.

Discussion and Conclusion

In the present study we demonstrate hTERT has a strong influence on the levels of osteopontin and osteocalcin mRNA during the osteogenic differentiation. The levels of osteopontin and osteocalcin weren't only increased but detected earlier than expected. Osteopontin and osteocalcin are the main proteins of the bone matrix and the up regulation of these genes in the osteoblasts could enhance the healing of damaged bone tissue. Studies need to be carried out in order to confirm if the increase on osteopontin and osteocalcin mRNA levels are translated to the protein levels as well. Our results are in accordance and support the hypothesis where the telomerase accelerates the osteogenic differentiation besides to extend the lifespan of mesenchymal stem cells15,16. Studies performed in mice shown that deficiencies in genome maintenance molecules, such as telomerase, are related to a low bone mass phenotype as result of impairment in osteogenic potential and osteoblast differentiation [3] .

Due the ability of telomerase trigger the expression of genes related with proliferation such as FGF and EFGR17 it has been suggested that the expression of hTERT in somatic cells could increase the risk of cancer. However, in germinative tissues, where telomerase can be detected in higher levels, there's no higher cancer incidence when compared to the other tissues18. Our results demonstrated that telomerized hMSC didn't develop into tumours in NUDE mice. Experimental studies on cell transplantation of genetically modified cells show that hTERT does not cooperate with known oncoproteins in tumorigenesis19. Nevertheless, there's much more to learn about telomerized cells before using them in the clinic.

In our work, we choose to deliver the hTERT DNA to human mesenchymal stem cells through an adenoviral vector. Adenovirus are among the most commonly used vectors for gene therapy because they are relatively easy to manipulate using recombinant DNA techniques and can accommodate large segments of DNA. Besides, adenoviruses are ubiquitous which means they can infect a broad range of different cells and finally their viral genome doesn't undergo rearrangement at high rates20. Although adenovirus can elicit immune responses, they have low pathogenicity in humans.

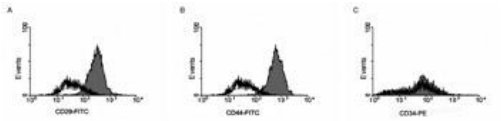
The influence of the telomerase in the osteogenic process is more complex and possibly involves the activation of more genes. Much about this interaction still needs to be elucidated. Despite of this, our findings strengths the concept of using telomerized hMSC to treat bone defects in aging.

Table 1: Semi-quantitative PCR primers

	Primer sequence 5'-3'	GI number
HPRT	*Forward*	4504482

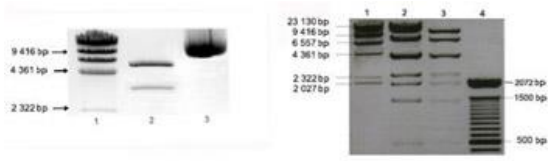
	TGAAGGAGATGGGAGGCCATCAC	
	Reverse AAATCCAACAAAGTCTGGCCTGTATCC	
Osteopontin	*Forward* CCCACAGACCCTTCCAAGTA	38146097
	Reverse AACCACACTATCACCTCGGC	
Osteocalcin	*Forward* TGTCCAAGCAGGAGGGCAG	41152108
	Reverse TTGAGCTCACACACCTCCCT	
hTERT	*Forward* CGGAAGAGTGTCTGGAGCAA	018167
	Reverse GGATGAAGCGGAGTCTGGA	

Figure 1



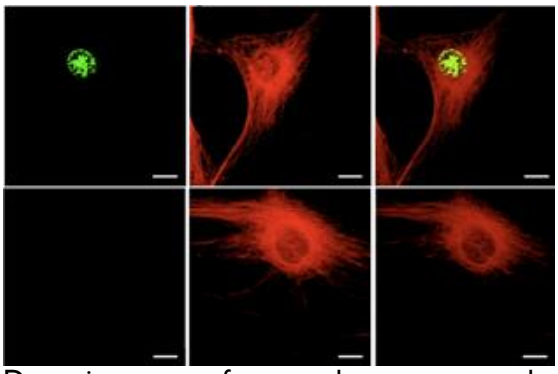
Flow cytometry analysis for CD29 (A), CD44 (B) and CD34 (C) on human mesenchymal stem cells. Representative picture of cells obtained from three different donors. Labelled cells (filled grey histograms) were plotted against the isotype controls (open black histograms).

Figure 2



Steps of hTERT subcloning into the Adenoviral DNA vector. The gel on the left shows the plasmid pCI-neo-hTERT after the double digestion NheI and NotI restriction enzymes. First lane is the DNA ladder, 2nd lane the hTERT fragment of approximately 3500 bp, 3rd lane non-digested plasmid pCI-neo-hTERT. The gel on the right exhibits the DNA pattern generated by the digestion of the pAdeno-hTERT with the restriction enzyme XhoI. The pattern shows that the DNA was cloned in the right restriction sites and that there's no DNA rearrangement. First lane is the DNA ladder, 2nd and 3rd lanes the pAdeno-hTERT digested with XhoI, 4th lane DNA ladder.

Figure 3



Detection of telomerase by immunofluorescence. Confocal images of the telomerase were collected 48 hours post-infection of primary human osteoblasts with adenovirus-hTERT. Isolated primary human osteoblasts were double-labelled with a rabbit monoclonal antibody anti-telomerase (top row in the right, in green) followed by secondary antibodies goat anti-rabbit Alexa 488-conjugated and mouse monoclonal antibody anti-a tubulin (top row in the middle, in red) with the secondary antibody goat anti-mouse Alexa 568-conjugated. In the top row on the right are the overlaid images. In the bottom row, primary non-infected osteoblasts were double-labelled with the same antibodies. As it can be observed, there's no expression of telomerase in these cells. Scale bar, 5 mm

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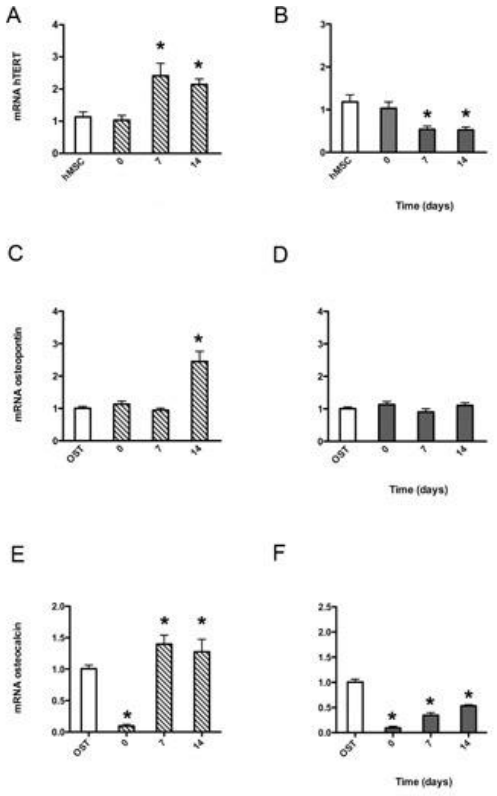
Figure 4



Graphic presentation of the data obtained with semi-quantitative PCR after 20 cycles. Data are expressed as fold change relative to hMSC (A and B) or to osteoblasts (C, D, E and F). Each experiment was carried out three times and for each sample the PCR was performed in triplicate. hMSC-human mesenchymal stem cells; OST-human primary osteoblasts; 0, 7 and 14 days of differentiation, * $p<0.05$

Figure 5

Tumorigenesis assay in NUDE mice. (A) NUDE mice 20 days after non-infected undifferentiated hMSC, (B) infected hMSC Ad-hTERT differentiated for 14 days and (C) MDAMB-231 (breast cancer) cells were injected subcutaneously in the right flank. The only group that developed tumours was the one injected with breast cancer cells.

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